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(57) **Abstract**

A mutant staphylococcal alpha hemolysin polypeptide containing a heterologous analyte–binding amino acid which assembles into an analyte–responsive heptameric pore assembly in the presence of a wild type staphylococcal alpha hemolysin polypeptide, digital biosensors, and methods of detecting, identifying, and quantifying analytes are described.

DESIGNED PROTEIN PORES AS COMPONENTS FOR BIOSENSORS

Statement as to Federally Sponsored Research

This invention was made with U.S. Government support under the Office of Naval Research grant No. N00014-93-1-0962. The government has certain rights in the invention.

Background of the Invention

The field of the invention is metal detection.

Biosensors are analytical devices that convert the concentration of an analyte into a detectable signal by means of a biologically-derived sensing element. Well-known biosensors include commercial devices for sensing glucose. In addition, true biosensors, biomimetric devices, and devices that use living cells have recently been developed. For example, to detect divalent metal cations, true biosensors have been made using the enzyme carbonic anhydrase (Thompson et al., 1993, Anal. Chem. 65:730-734), the metal binding site of which has been altered (Ippolito et al., 1995, Proc. Natl. Acad. Sci. USA 92:5017-5020). To monitor HIV antibody levels, the enzyme alkaline phosphatase into which an HIV epitope has been inserted has been utilized (Brennan et al., 1995, Proc. Natl. Acad. Sci. USA 92:5783-5787).

Summary of the Invention

The invention features a mutant staphylococcal alpha hemolysin (αHL) polypeptide containing a heterologous metal-binding amino acid. The polypeptide assembles into a heteroheptameric pore assembly in the presence of a wild type (WT) αHL polypeptide. Preferably, the metal-binding amino acid occupies a position in a transmembrane channel of the

133, 135, 137, 139, 141, 143, 145, 147 or 149. Alternatively, the heterologous amino acids are located on the outside of the transmembrane channel, i.e., the amino acids occupy two or more of the following positions of SEQ ID NO:1: 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148. The polypeptide may contain at least three non-consecutive heterologous metal-binding amino acids in the stem domain. Preferably, the polypeptide contains at least 4 non-consecutive heterologous metal-binding amino acids in the stem domain; more preferably, the amino acids occupy positions 123, 125, 133, and 135 of SEQ ID NO:1; more preferably, each these positions are occupied by the heterologous metal-binding amino acid His; and most preferably, the polypeptide is the αHL mutant 4H, as described below.

To facilitate separation and purification of mutant analyte-responsive αHL polypeptides, the polypeptide may also contain a heterologous amino acid, e.g., a Cys residue, at a site distant from the stem domain, e.g., at position 292 of SEQ ID NO:1.

The invention also features a heteromeric pore assembly containing a metal-responsive (M) αHL polypeptide, e.g., a pore assembly which contains a wild type (WT) staphylococcal αHL polypeptide and a metal-responsive αHL polypeptide in which a heterologous metal-binding amino acid of the metal-responsive αHL polypeptide occupies a position in a transmembrane channel of the pore structure. For example, the ratio of WT and M αHL polypeptides is expressed by the formula $WT_{7-n}M_n$, where n is 1, 2, 3, 4, 5, 6, or 7; preferably the ratio of αHL polypeptides in the heteroheptamer is $WT_{7-n}4H_n$; most preferably, the ratio is $WT_6 4H_1$. Homomeric pores in which each subunit of the heptomer is a mutated

mixture current signature with the standard current signature indicates the identity of the unknown analyte in the mixture.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims. All references cited herein are incorporated by reference in their entirety.

Brief Description of the Drawings

Fig. 1A is a diagram showing the interpretation of a digital/stochastic response of a single channel (patch clamp) recording using an analyte-responsive αHL pore assembly (the average upspike time durations →K_1, the analyte identity; the average downspike durations →[●], the analyte concentration).

Fig. 1B is a series of graphs of digital single channel recordings showing metal-responsiveness of an αHL pore assembly at various concentrations of Zn(II).

Fig. 1C is a diagram of the structure of a heteromeric αHL pore ($WT_6 4H_1$) assembly showing a Zn(II) binding site with a view of the heptamer perpendicular to the seven-fold axis of the pore. The top of the structure is on the *cis* side of the membrane in bilayer experiments. The 14-strand β barrel at the base of the structure opens the lipid bilayer. In the 4H subunit, residues Asn123, Thr125, Gly133, and Leu135 were replaced with histidine and Thr292 with cysteine. A close-up view of the antiparallel β strands that contribute to the lower part of the barrel is shown in Fig. 1E below.

Fig. 1D is a diagram of the structure of a heteromeric αHL pore ($WT_6 4H_1$) assembly showing a Zn(II) binding site with a view of the heptamer down the seven-fold axis from the top (*cis* side) of the pore. The four heterologous histidinyl residues project into the lumen of the channel, while Cys292 is distant from the channel

Fig. 2C is a photograph of an electrophoretic gel showing separation of different classes of α heptamers. WT αHL and the mutant 4H, both [^{35}S]-labeled, were mixed in the ratios indicated, allowed to assemble on rRBCM and then treated as shown in Fig. 2B. The membranes were solubilized in gel loading buffer containing SDS and, without heating, subjected to electrophoresis in a 7% gel. A phosphorimager display of the molecules migrating near the 200 kDa marker (myosin heavy chain) is shown. The observed ratios of oligomer classes seen in each lane approximate those shown in Fig. 2A. The lane marked "All" contained a mixture of the solubilized samples at all five WT:4H ratios.

Fig. 3A is a photograph of an electrophoretic gel showing purified αHL heteroheptamers. Heptamers were stable in SDS and the subunits did not interchange. All eight radiolabeled $WT_{n-m}4H_m$ heptamers were purified by SDS-PAGE, rerun on a 40 cm long 8% SDS-polyacrylamide gel and visualized by autoradiography. The individual heteromer species (lanes 1-8) retained their relative mobilities, resulting in the staircase appearance of the image.

Fig. 3B is an electrophoretic gel showing that WT_7 and $4H_7$ did not become scrambled under the conditions used for extraction, storage and reconstitution. An excised WT_7 band was mixed and coeluted with an excised $4H_7$ band. The sample was kept at 4°C for 24 h and then stored at -20°C. The thawed sample was run on a 40 cm long 8% SDS polyacrylamide gel. The bands retained their integrity (i.e. there is no ladder of species to suggest subunit interchange).

Fig. 3C is a photograph of an electrophoretic gel showing the ratio of the WT and 4H subunits in each purified heptamer. Heptamers were made as described in the legend to Fig. 3A. Half of each sample was subjected to electrophoresis without heating (top panel), while the

Zn(II) to the *trans* chamber results in discrete fluctuations between two open states, the original state (-28.4 pA) and another of -25.7 pA (mean = -24.4 ± 1.8 pA, n = 7). The ratio of the conductance of the new state to the conductance of the original state (g/g_o) was 0.93 ± 0.01 (n = 7).

Fig. 4D is a series of graphs of digital single channel recordings showing the dependence of the partial channel block of the heteromeric pore $WT_6 4H_1$ on Zn(II) concentration. Single-channel current recordings were made at various *trans* free Zn(II) concentrations. A solution containing 1 M NaCl, 50mM MOPS, pH 7.5, Zn(II) was buffered with 100μM pyridine-2.6-dicarboxylic acid and 10μM EDTA. All points amplitude histograms are shown below each graph. The histograms can be fitted to the sum of two Gaussian functions, suggesting two distinct states: (i) the fully open channel as seen in the absence of Zn(II), (ii) the partly closed, g/g_o = 0.93, Zn(II) dependent substrate. The normalized areas of the Gaussian functions represent the occupancy of each state at the displayed Zn(II) concentration. When the openings or closing are short, the amplitudes of the transitions are underestimated, resulting in shifts of the peaks to lower values, for example, for 190nM Zn(II).

Fig. 5A is a series of graphs of digital single channel recordings from $WT_6 4H_1$ in the presence of 5 μM free Zn(II) or 5μM free Co(II) showing response of the heteromeric pores to different M(II)s and tuning of the sensitivity to M(II)s by adjustment of subunit composition. Top, transmembrane potential -40 MV; bottom, transmembrane potential +40 mV.

Fig. 5B is a series of graphs showing the response of pores containing more than one 4H subunit to Zn(II). $WT_5 4H_2$ (concentration of free Zn(II) = 50 μM), $WT_7 4H_3$ (20 μM) and $4H_7$ (10μM). Left, digital single channel

the ion flux through the αHL pore assembly/channel in a membrane separating two liquid phases).

The compositions, devices and methods described herein can be used to track diverse analytes of interest in spatio-temporal gradients in water, in sediments and in the air. Such a capability would permit, for example, gradiometer-directed locomotion of robots. Other uses include detection, identification, and quantification of analytes in the environment, e.g., Cu, Zn, or Ni in effluents from underwater and dry dock hull cleaning operations, in shipboard waste processing, and in ocean micronutrient analyses.

Biosensors which incorporate protein pores as sensing components have several advantages over existing biosensors. In particular, bacterial pore-forming proteins, e.g, αHL, which are relatively robust molecules, offer all the advantages of protein-based receptor sites together with an information-rich signal obtained by single-channel recording.

αHLis a 293 amino acid polypeptide secreted by *Staphylococcus aureus* as a water-soluble monomer that assembles into lipid bilayers to form a heptameric pore. The heptamer is stable in sodium dodecyl sulfate (SDS) at up to 65°C. The biophysical properties of αHL altered in the central glycine-rich sequence, by mutagenesis or targeted chemical modification, demonstrate that this part of the molecule penetrates the lipid bilayer and lines the lumen of the transmembrane channel. The channel through the heptamer is a 14-strand β barrel with two strands per subunit contributed by the central stem domain sequence (spanning approximately amino acids 110-150 of SEQ ID NO:1).

simultaneously using the compositions and biosensor devices described herein. Selectivity is not a problem because a single analyte binding site can only be occupied by a single analyte at one time. Analyte-responsive αHL pores have been successfully used to detect an analyte of interest, e.g., a metal ion, in a solution containing a mixture of analytes as well as in solutions containing various concentrations of a single analyte.

Digital/Stochastic Single Channel Biosensors Using Analyte-Responsive αHL Polypeptides

The attainment of sensitivity and selectivity is a major problem with most known biosensors as they are based on an integrated signal from numerous sensor molecules. The resulting signal is analogue/steady state and contains limited information about analyte identity(ies) and concentration(s). Analogue/steady state detection data is extremely difficult to extract reliably, even by modern processing hardware and software. For example, simultaneous competition for an analyte-binding site by many different analytes is a major problem. This problem is solved by the analyte-responsive αHL pores described herein.

The disclosed analyte-responsive αHL compositions are unique. A biosensor using an analyte-responsive αHL as the sensing component is tunable to any analyte target of interest by introducing an analyte-binding site directly into a measurable channel. Biosensors which incorporate an analyte-responsive αHL pore assembly reliably detect analytes in single channel mode, i.e., an individual analyte is detected as it randomly (stochastically) hops on and off a single binding site. These events are detected as modifications or perturbations of the ion conductance in the single channel.

exhibit distinct responses to M(II)s. Analyte-responsive αHL pores were generated through subunit diversity and combinatorial assembly.

Sensor arrays with components with overlapping analyte specificity, i.e., pore assemblies made from αHL polypeptides which respond to a variety of analytes, e.g., metal ions, provide a yet more powerful means for the simultaneous determination of multiple analytes and to expand the dynamic range. By using the design principles disclosed herein, binding sites for diverse analytes, e.g., different metal ions, can be engineered into the lumen of the transmembrane channel of an heteromeric αHL pore assembly or near an entrance to the transmembrane channel, e.g., near the cis entrance of the channel. The digital/stochastic detection mode can be generalized to classes of proteins other than pore-forming proteins, e.g., receptors, antibodies, and enzymes, with attached fluorescent probes to monitor individual binding events using imaging technology directly analogous to single channel recording. For example, analyte binding and dissociation from an active site (e.g., naturally-occurring or re-engineered analyte-binding site) of a remodeled fluorescent-tagged antibody, lectin, or enzyme is detected using the detection methods described above to determine the presence and/or concentration of an antigen, carbohydrate moiety, or enzyme ligand, respectively.

The compositions and biosensor devices described herein offer sensitivity, speed, reversibility, a wide dynamic range, and selectivity in detecting and determining the identity and concentration of analytes such as metal ions. αHL pores, remodeled so that their transmembrane conductances are modulated by the association of specific analytes, make excellent components of biosensors.

αHL pore assemblies

WT αHL pores are homomeric; that is, all seven subunits are the same. The analyte-responsive pores described herein may be homomeric or heteromeric and contain at least one mutated αHL polypeptide subunit. For example, a pore assembled from seven subunits has the formula $WT_{7-n}MUT_7$, where MUT is a mutant αHL polypeptide and where n = 1, 2, 3, 4, 5, 6, or 7. Preferably, the MUT subunit is an analyte-binding αHL polypeptide. The amino acid sequence of MUT differs from that of WT in that MUT may be longer or shorter in length compared to the WT subunit (e.g., MUT may be truncated, contain internal deletions, contain amino acid insertions, or be elongated by the addition terminal amino acids, compared to the WT sequence); alternatively, MUT may contain one or more amino acid substitutions in the WT sequence (or MUT may differ from WT both in length and by virtue of amino acid sequence substitutions). The engineered changes in the MUT subunit preserve the ability of MUT to associate with other αHL polypeptides to form a pore structure.

A heteromeric pore was made that binds the prototypic analyte Zn(II) at a single site in the lumen of the transmembrane channel, thereby modulating the single-channel current. In addition, M(II)s other than Zn(II) modulate the current and produce characteristic signatures. Heteromers containing more than one mutant subunit exhibit distinct responses to M(II)s. The invention therefore provides an extensive collection of heteromeric responsive pores suitable as components for biosensors.

Molecular modeling of αHL pore assemblies

The three-dimensional structure of an αHL pore assembly was determined using known methods, e.g., those described in Song et al., 1996, Science 274:1859-1865.

present in αHL-RL: Val124→Leu, Gly130→Ser, Asn139→Gln and Ile142→Leu. The region encoding amino acids 118-138 was removed by digestion with *BsiWI* and *Apa*l and replaced with two synthetic duplexes (*BsiWi-Spe*l and *Spe*l-*Apa*l) encoding the replacements Asn123→His, Val124→Leu, Thr125→His, Gly130→Ser, Gly133→His, Leu135→His. A 700 base pair fragment of the resulting construct, encompassing the four new histidines, was removed with *Ndel* and *Mfel* and used to replace the corresponding sequence in αHL-Thr292→Cys. The entire coding region of the resulting αHL-4H/Thr292→Cys construct was verified by sequence analysis.

Expression and purification of αHL polypeptides

Monomeric WT-αHL was purified from the supernatants of *S. aureus* cultures using known methods, e.g., the method described in Walker et al., 1992, J. Biol. Chem. 267: 10902-10909. [^{35}S]-Methionine-labeled WT-αHL and αHL-4H were obtained by coupled *in vitro* transcription and translation (IVTT). Separate reactions conducted with a complete amino acid premix and the premix without unlabeled methionine were mixed to yield a solution containing αHL at > 10μg/ml. αHL in the IVTT mix was partially purified by (i) treatment with 1% (w/v) polyethyleneimine (PEI) to precipitate nucleic acids, (ii) treatment with SP Sephadex C50, pH 8.0 (to remove the residual PEI), and (iii) binding to S-Sepharose Fast Flow at pH 5.2, followed by elution with 10mM sodium acetate, pH 5.2, 800mM NaCl. The concentration of αHL (in the IVTT mix or after the purification) was estimated by a standard quantitative hemolytic assay.

Oligomerization of αHL polypeptides

WT and αHL-4H were mixed in various molar ratios (6:0, 5:1, 1:1, 1:5, and 0:6) and allowed to oligomerize on rabbit erythrocyte membranes, liposomes, and other planar bilayers. The αHL polypeptides self-assemble into

USA; 1.5 μl of 10mg/ml). The latter had been bathed sonicated at room temperature until clear (30 min) in 10mM MOPS, pH7.4, 160mM NaCl. The mixture (60 μl) was then treated with 2 M TAPS, pH 8.5 (10 μl), and 10mM DTT (6 μl) for 10 min at room temperature, followed by 100mM IASD (5 μl in water) for 60 min at room temperature. Gel loading buffer (5x, 25 μl) was then added, without heating, and a portion (50 μl) was loaded into an 8 mm wide lane of a 40 cm long, 1.5 mm thick 6% SDS-polyacrylamide gel, which was run at 4°C at 120 V for 16h, with 0.1mM thioglycolate in the cathode buffer. The unfixed gel was vacuum dried without heating onto Whatman 3MM chromatography paper (#3030917).

Each of the eight heptamer bands was cut from the gel, using an autoradiogram as a guide. The excised pieces were rehydrated with water (100 μl). After removal of the paper, each gel strip was thoroughly crushed in the water and the protein was allowed to elute over 18 h at 4°C. The solvable eluted protein was separated from the gel by centrifugation through a 0.2 μm cellulose acetate filter (#7016-024, Rainin, Woburn, MA, USA). A portion (20 μl) was saved for single channel studies. Sample buffer (5x, 20 μl) was added to the rest of each sample. Half was analyzed, without heating, in a 40 cm long 8% SDS-polyacrylamide gel. The other half was dissociated at 95°C for 5 min for analysis of the monomer composition in a 10% gel.

<u>Biosensor: planar bilayer recordings</u>

Detection of analytes using heteroheptameric αHL pore assemblies in planar bilayers was carried out as follows. A bilayer of 1,2-diphytanoyl-sn-glycerophosphocholine (Avanti Polar Lipids) was formed on a 100-200 μm orifice in a 25 μm thick teflon film (Goodfellow Corporation, Malvern, PA, USA), using standard methods, e.g., the method of Montal and Mueller

domain) to form a cluster of imidazole sidechains. αHL polypeptides in which heterologous metal-binding amino acids have been introduced such that they are located on the outside of the barrel (e.g.., at even numbered positions of the stem domain) of the pore assembly also confer responsiveness to metal ions. In addition, amino acid substitutions in regions of the αHL polypeptide outside the stem domain but which are close to the lumen of the transmembrane channel, e.g., at the mouth of the channel, also confer metal responsiveness.

The channel through the heptamer is a 14-strand β barrel with two strands per subunit (see Figs. 1C-F) contributed by the central stem domain sequence which spans approximately amino acids 110-150 of SEQ ID NO:1: EYMSTLTYGF NGNVTGDDTG KIGGLIGANV SIGHTLKYVQ (SEQ ID NO:2). Structural data indicates that the β barrel is sufficiently flexible for at least three sidechains to act as ligands to Zn(II) in the preferred tetrahedral configuration.

To facilitate separation of polypeptides, the 4H polypeptide was also cogged by chemical modification of the single cysteine (at position 292) with 4-acetamido-4'-[(iodoacety)amino]stilbene-Z,Z'-disulfonate (IASD). The Cys-cogged αHL (Thr292→Cys; without amino acid substitutions in the stem domain) modified with IASD forms fully active homomers. This modification caused an incremental increase in the electrophoretic mobility of heptamers in SDS-polyacrylamide gels allowing heteromers to be easily separated from each other and from wild-type (WT) heptamers. Each disulfonate made an approximately equal contribution to the mobility, which is independent of the arrangement of the subunits about the seven-fold axis. The chemical modification was distant from the stem domain of the polypeptide which lines the channel of the heteromeric pore assembly.

containing the homomers WT_7 and IASD-modified $4H_7$, were mixed and taken through the elution and storage procedures before re-electrophoresis, which again indicated no scrambling (Fig. 3B). Furthermore, the eluted heptamers were free of residual proteins from the IVTT mix, as determined by silver staining. Finally the ratio of the αHL polypeptides in each of the heteromeric pore assemblies examined was as expected, when determined by quantitative analysis of radio-labeled polypeptides from purified heteromers dissociated by heating to 95°C (Fig. 3C). The electrophoretic gel shown in Fig. 4A confirms the heteromeric channel structure of the αHL pore assembly.

Digital single-channel currents from heteromeric metal-responsive pores

The properties of $WT_6 4H_1$ were examined by digital single-channel recording in a planar bilayer biosensing apparatus. Methods for forming planar bilayers in biosensors are known in the art, e.g., Hanke et al., 1993, Planar Lipid Bilayers, Academic Press, London, UK or Gutfreund, H., 1995, Kinetics for the Life Sciences, Cambridge University Press, Cambridge, UK. In this experiment, a lipid bilayer was formed across an aperture (100-200μm diameter) in a teflon film (25μm thick) that separates two chambers (2ml each) containing electrolyte. With a potential applied across the bilayer, the ion flux through single αHL pores was measured with a sensitive, low-noise amplifier.

To obtain single-channel currents, the eluted heptamers were added at high dilution (typically 1:1000) to the *cis* chamber of the bilayer apparatus to a final concentration of 0.02-0.1ng/ml (Figs. 4B-D). $WT_6 4H_1$ exhibited a partial and reversible channel block (g/g_0-$0.93 \pm 0.01_1$; n=7) in the presence of 50μM Zn(II) in the

existence of mutants with proline residues in the central domain that form pores.

The conductance of WT_7 pores (675±62pS_1 1M N_aCl_1 50mM MOPS, pH7.5_1-40mV_1 n=8) was similar to that of $WT_6$4H_1 in the absence of Zn(II) (660±40pS_1 n=7). The conductance of $WT_6$4H_1 with Zn(II) bound was reduced to 610±45 pS (n=7). A partial channel block may be due to a simple physical blockade, distortion of the barrel, or electrostatic effects.

Figs. 4C and 4D show digital responses of the engineered $WT_6$4H_1 hybrid channel to various levels of Zn(II). The digital pattern is due to the stochastic (random) effect of single zinc ions hopping on and off the tetra-histidyl binding site engineered into the lumen of the transmembrane channel of an αHL pore assembly. The two channel states are open (Zn(II) off, 100% open) and gated (Zn(II) on, 93% open). Average time in the open state is the reciprocal of bimolecular rate constant x [Zn(II)], from which Zn(II) is quantified, while average time in the gated state is the reciprocal of the first order off constant (the analyte signature or identity). Monovalent metal cations gave no signal. These data indicate that the metal-responsive αHL polypeptides and pore assemblies used as components of a biosensor provide a means to achieve unambiguous analyte identity and concentration(s). Existing chemo/bio-sensors are analog/steady state, whereas the channel of the αHL pore assembly is digital/stochastic. Fig. 4 also shows that αHL pore assemblies have an wide dynamic range of analyte detection (at least 10,000-fold in analyte concentration. Even at very low fractional site occupancies, the signal (being digital and not analog) is not degraded. At very low site occupancy, it simply may take longer to collect to collect data

conventional chemo/bio-sensors, requiring extensive down-stream processing. In contrast, the identity and concentration of analytes can easily, reliably, and accurately determined from traces such as those in Figs. 4A-D and 5A-B, i.e., analytes can be identified (as well as quantified) by the single-channel current signature (Δg, k_{on}, k_{off}, voltage dependence of these parameters). Fig. 5 also illustrates that the channel can further be tuned by changing the transmembrane voltage. Figs. 7A-B show that digital output patterns corresponding to different analytes allow the detection and quantification of analytes, e.g., Zn (II) and Ni (II), even in solutions containing a mixture of analytes. These data indicate that αHL biosensors may be used to detect, identify, and quantify analytes in complex mixtures, e.g., environmental samples or waste water samples.

Additional 4H heteromers exhibit different responses to divalent cations

Structural variants of αHL pores resulting from combinatorial assembly provide yet another means by which to tune an αHL channel for detection of analytes. In addition to the experiments described above, other combinations of $WT_{7-n}4H_n$ were tested. The extent of single-channel block by Zn(II) increased with the number of 4H subunits. Multiple subconductance states were observed as exemplified by the data for WT_54H_2, WT_44H_3, and $4H_7$ (Fig. 5B). The specific permutations of the WT_54H_2 and WT_44H_3 pores in these recordings was not determined, however single-channel recording actually provides a means to "separate" the various permutations of each combination of heteromers. According to these data, combinatorial assembly can provide pores with characteristic responses over a wide range of analyte concentrations.

8. The polypeptide of claim 5, wherein said polypeptide comprises at least three non-consecutive heterologous amino acids in the stem domain of said polypeptide.

9. The polypeptide of claim 5, wherein said polypeptide comprises at least 4 non-consecutive heterologous amino acids in the stem domain of said polypeptide.

10. The polypeptide of claim 9, wherein said amino acids occupy positions 123, 125, 133, and 135 of SEQ ID NO:1.

11. The polypeptide of claim 10, wherein said polypeptide is 4H.

12. The polypeptide of claim 1, wherein said amino acid is selected from the group consisting of Ser, Thr, Met, Trp, and Tyr.

13. The polypeptide of claim 12, wherein said amino acid is selected from the group consisting of Glu, Asp, Cys, His.

14. The polypeptide of claim 13, wherein said amino acid is His.

15. A staphylococcal alpha hemolysin (αHL) polypeptide comprising at least two non-consecutive heterologous amino acids in a stem domain of said polypeptide, wherein each of said heterologous amino acids binds an organic molecule.

24. The pore assembly of claim 21, wherein said pore assembly is a heptamer having the formula $WT_{7-n}M_n$, wherein n is greater than zero and less than seven.

25. The pore assembly of claim 17, wherein said analyte-binding αHL polypeptide is 4H.

26. The pore assembly of claim 21, wherein said analyte-binding αHL polypeptide is 123W/125W.

27. The pore assembly of claim 25, wherein the pore assembly is a heptamer having the formula $WT_{7-n}4H_n$.

28. The pore assembly of claim 27, wherein the pore assembly is a heteroheptamer having the formula $WT_6 4H_1$.

29. A digital biosensor device comprising the pore assembly of claim 21.

30. The device of claim 29, wherein said analyte-binding αHL polypeptide comprises at least two non-consecutive heterologous amino acids in the stem domain, wherein each of said heterologous amino acids binds a metal.

31. The device of claim 29, wherein said analyte-binding αHL polypeptide comprises a chelating molecule in the stem domain of said polypeptide.

32. The device of claim 29, wherein said device detects binding of a metal ion to said analyte-binding αHL polypeptide.

40. A method of identifying an unknown analyte in a mixture of analytes comprising,

(a) contacting said mixture with the pore assembly of claim 21;

(b) detecting an electrical current in a digital mode through two or more channels to determine a mixture current signature;

(c) comparing said mixture current signature to a standard current signature of a known analyte, wherein a concurrence of said mixture current signature and said standard current signature indicates the identity of said unknown analyte in said mixture.

41. The method of claim 40, wherein each of said known and unknown analytes is a metal ion.

42. A method of identifying an analyte in a mixture of analytes comprising,

(a) contacting said mixture with the pore assembly of claim 21;

(b) detecting a single channel current in a digital mode to determine a mixture current signature;

(c) comparing said mixture current signature to a standard current signature of a known analyte, wherein a concurrence of said mixture current signature and said standard current signature indicates the identity of said unknown analyte in said mixture.

43. The method of claim 42, wherein each of said unknown and known analytes is a metal ion.

44. The method of claim 43, wherein said metal ion is Zn(II).

45. The method of claim 43, wherein said metal ion is Co(II), Cu(II), Ni(II), or Cd(II).

FIG. 1C

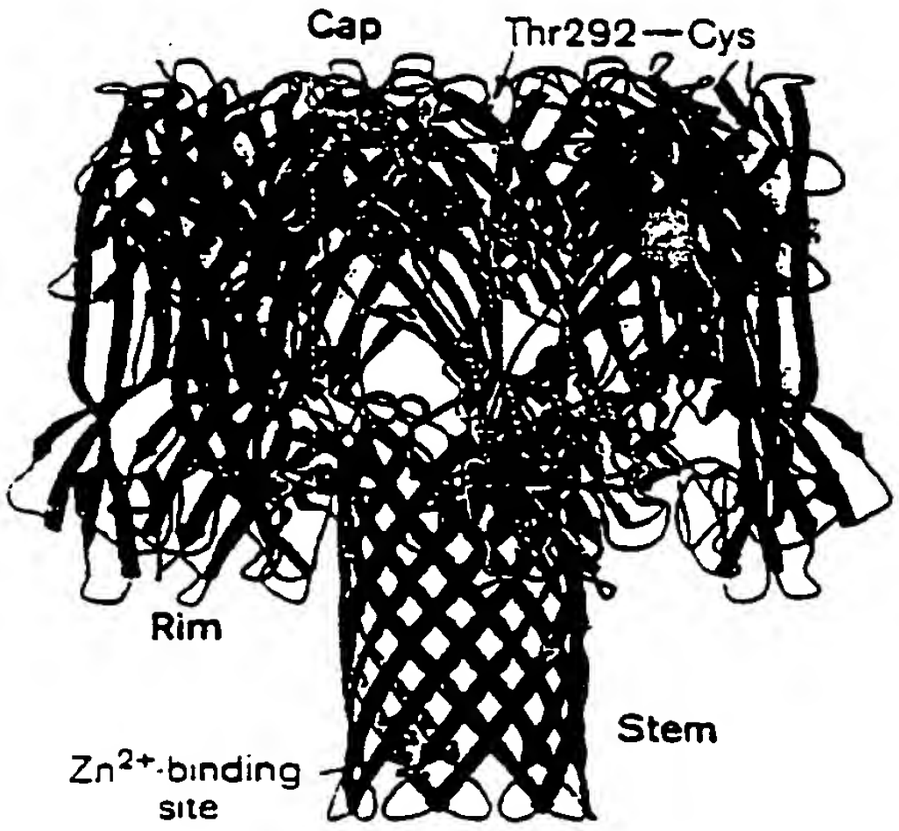


FIG. 1D

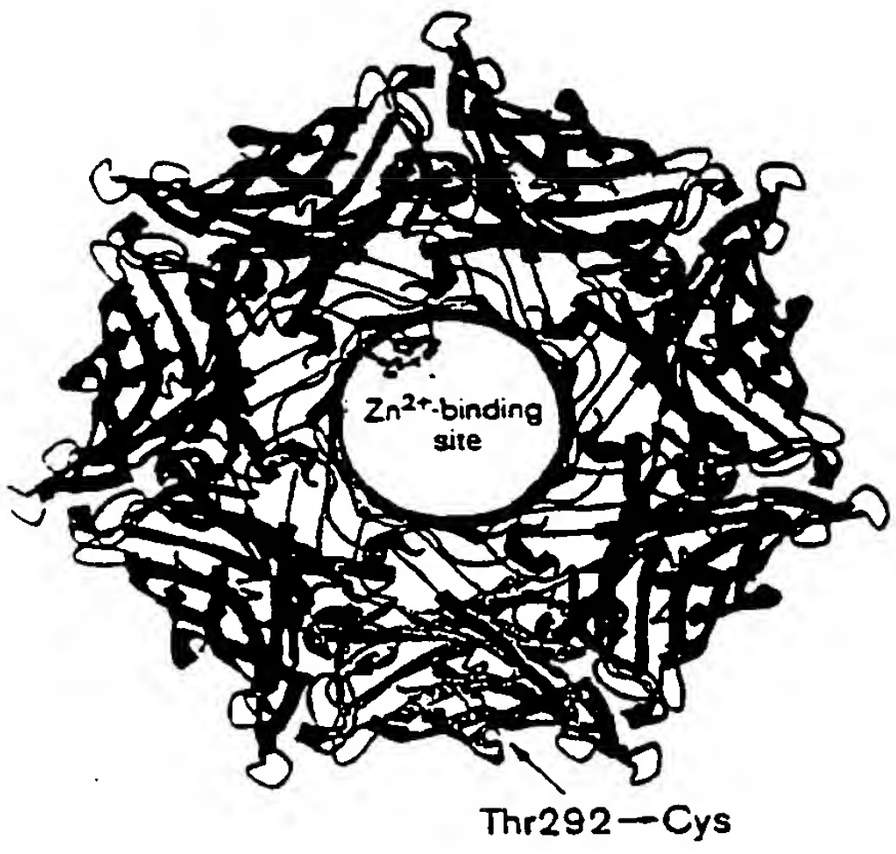


FIG. 1E

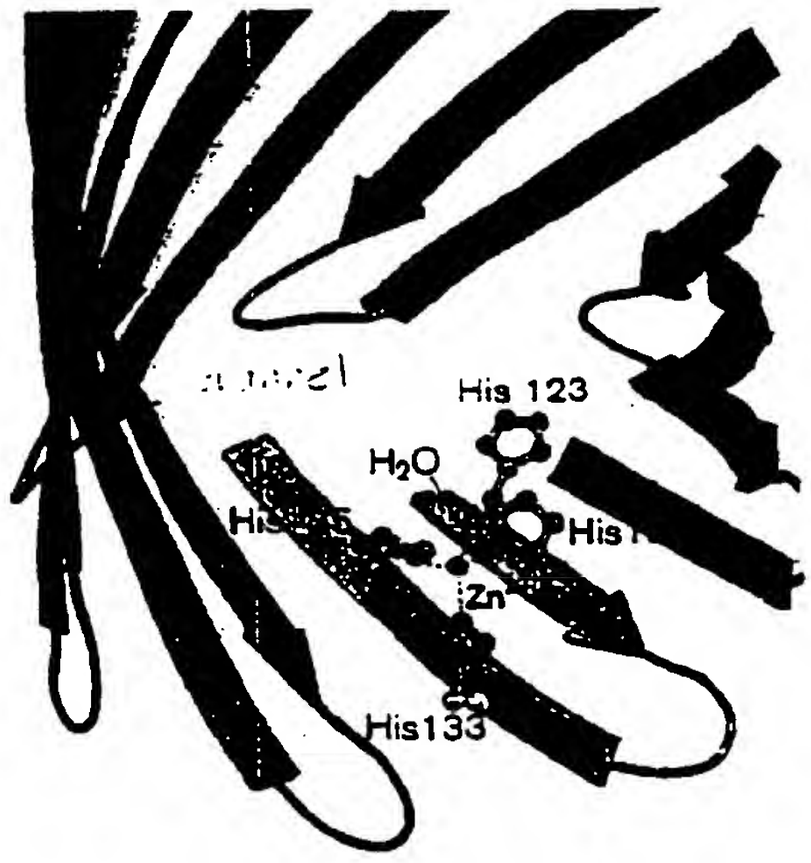


FIG. 2A

Possible permutations	Percent occurence at mixing ratio of:		
	5:1	1:1	1:5
WT,	27.9	0.78	0.0003
WT,MUT,	39.1	5.47	0.013
WT,MUT,	23.4	16.4	0.19
WT,MUT,	7.81	27.3	1.56
WT,MUT,	1.56	27.3	7.81
WT,MUT,	0.19	16.4	23.4
WT,MUT,	0.013	5.47	39.1
MUT,	0.0003	0.78	27.9

FIG. 2B

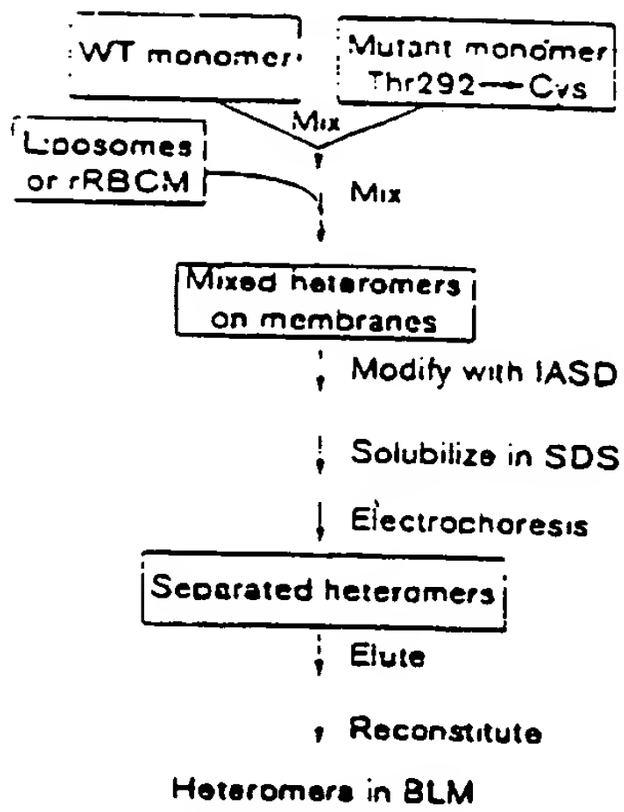


FIG. 2C

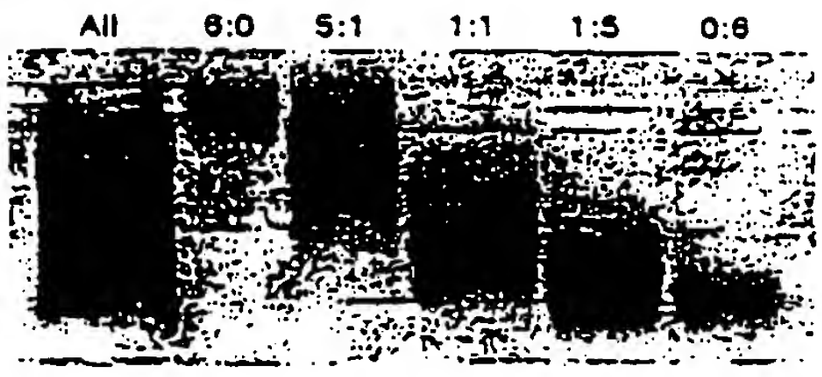


FIG. 4A

FIG. 4B

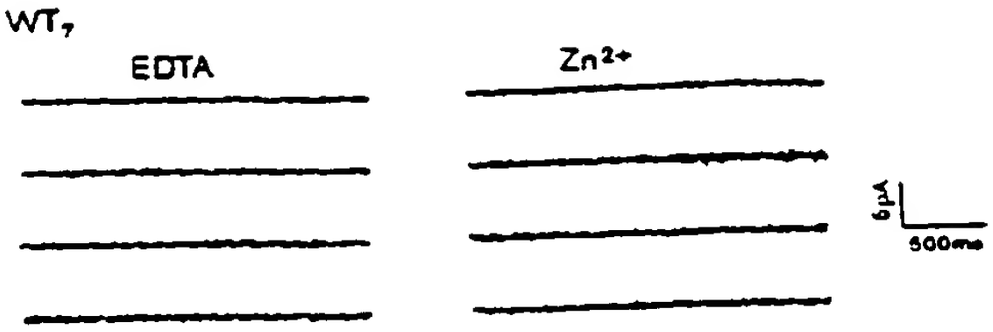


FIG. 4C

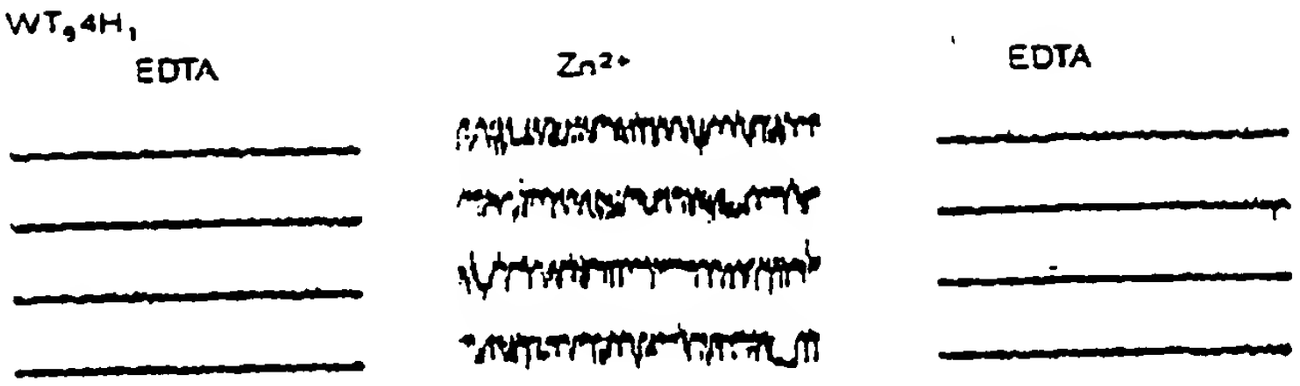


FIG. 4D

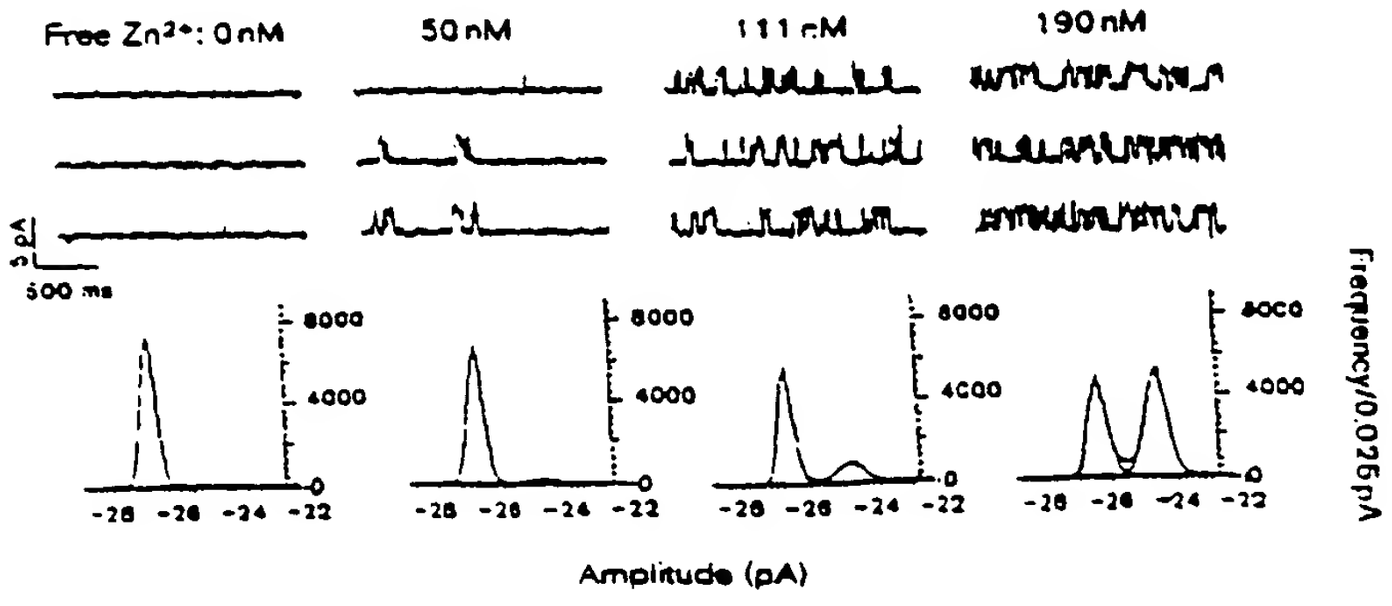
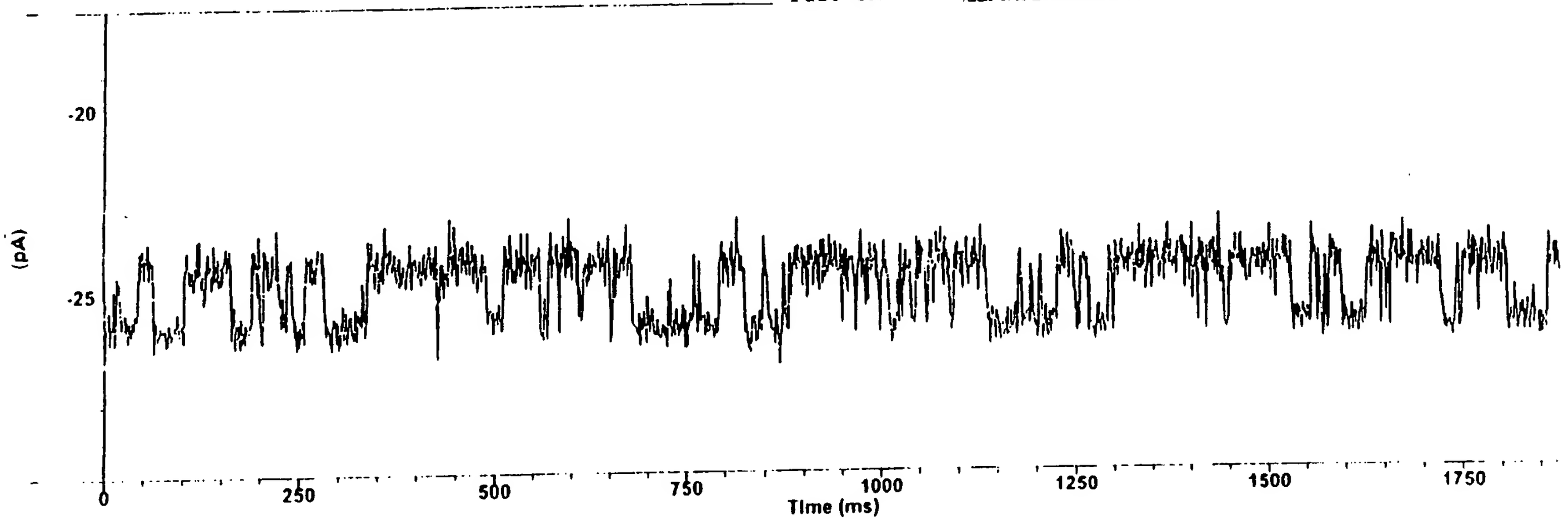


FIG. 6A



expanded in Fig 6B

— 40 mV
150 mM Zn (i)

FIG. 7A

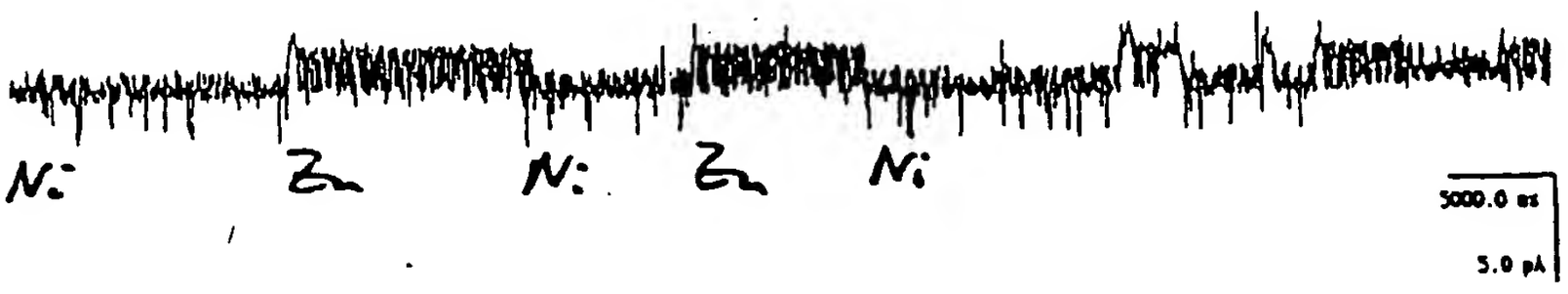
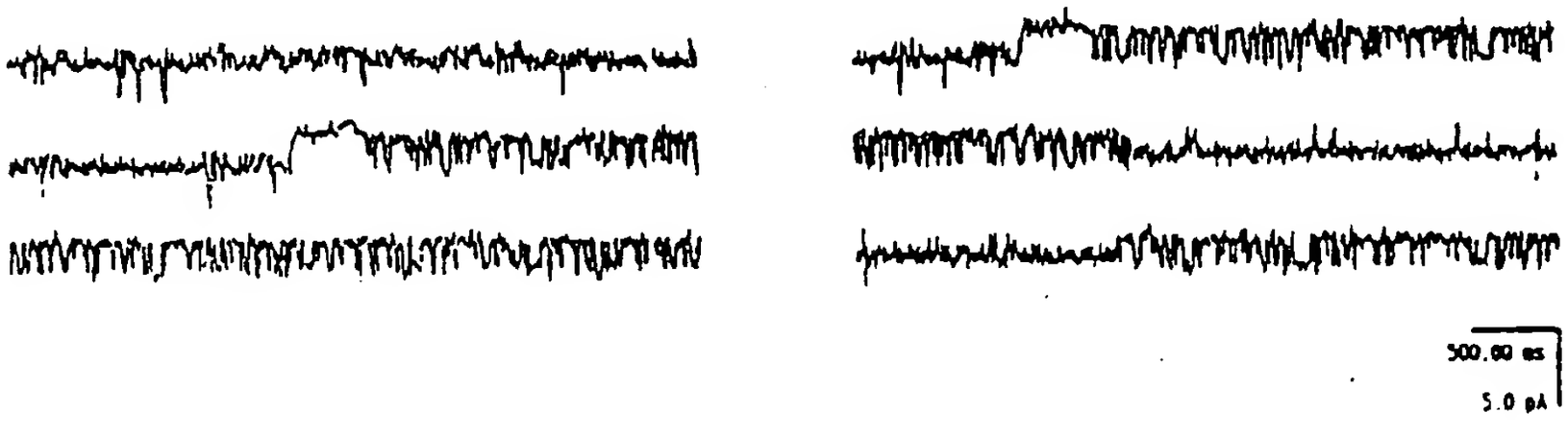


FIG. 7B



$WT_6 4H_1$

-40 mV

40 + 40 mM

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15354

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 14/195, 14/305, 14/31; G01N 33/20, 33/48
US CL :435/7.2; 436/73; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.2; 436/73; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, BIOSCIENCE, BIOSIS, CAPLUS, MEDLINE

search terms: alpha hemolysin, staphylococ?, muta?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P --- A, P	US 5,777,078 A (BAYLEY et al.) 07 July 1998, columns 1-4, 7-9, 16, 21, and 26.	1-4, 13, 14, 21, 22, 23, 24, 29 --- 5-12, 15-20, 25-28, 30-45

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
14 OCTOBER 1998	02 DEC 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MICHAEL D. PAK Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15354

BOX I. OBSERVATIONS WHERE CLAIMS WERE FOUND UNSEARCHABLE

2. Where no meaningful search could be carried out, specifically:

The claims are unsearchable to the extent that they require reference to the specified sequences from the sequence listing. Because Applicant has not furnished a machine-readable copy of the sequence listing as required by PCT Rule 5.2, no meaningful search of the sequences per se can be carried out by this Authority. However, the subject matter of the claims has been searched to the extent possible with reference to the balance of the description.